Form PTO-1390 (REV. 11-2000) U S DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE	ATTORNEY'S DOCKET NUMBER 9286-9
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371	U S APPLICATION NO (If known, see 37 CFR 1 5) **10/070144**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/EP00/07796	10 August 2000	3 September 1999

TITLE OF INVENTION
Method for Screening of Inhibitors of the Biosynthesis of Riboflavin

APPLICANT(S) FOR DO/EO/US
BACHER, Adelbert; MÖRTL, Simone

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include items (5), (6), (9) and (21) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4)
7. ☐ Amendments to the claims of the International Application Under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report Under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11 to 20 below concern document(s) or information included:

11. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
14. ☐ A SECOND or SUBSEQUENT preliminary amendment.
15. ☐ A substitute specification.
16. ☐ A change of power of attorney and/or address letter.
17. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
18. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
19. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4)
20. ☒ Other items or information: International Search Report; IPER

U.S. APPLICATION NO (if known, see 37 CFR 1.5) 10/070144	INTERNATIONAL APPLICATION NO PCT/EP00/07796	ATTORNEY DOCKET NO. 9286 9

21. ☐ The following fees are submitted: BASIC NATIONAL FEE (37 CFR 1.492(a) (1) - (5)):	CALCULATIONS	PTO USE ONLY
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1 445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO..... **$1040.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO..... **$890.00**		
International preliminary examination fee 37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO..... **$740.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)..... **$710.00**		
International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)..... **$100.00**		
ENTER APPROPRIATE BASE FEE AMOUNT =	$890.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	13 - 20 =	0	x **$18.00**	$	
Independent Claims	4 - 3 =	1	x **$84.00**	$84.00	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)			+ **$280.00**	$	

TOTAL OF ABOVE CALCULATIONS =	$974.00	
☐ Applicant claims small entity status. See 37 CFR 1.27. The fees indicated above are reduced by 1/2 -	$	
SUBTOTAL =	$974.00	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492(f)). +	$	
TOTAL NATIONAL FEE =	$974.00	
Fee for Recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$	
TOTAL FEES ENCLOSED =	$974.00	
	Amount to be refunded:	$
	charged:	$

a. ☒ A check in the amount of $974.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. 50-0220 in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☐ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 50-0220. A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

20792
PATENT TRADEMARK OFFICE

Ken Sibley
Kenneth D. Sibley

Date: March 1, 2002

CERTIFICATE OF EXPRESS MAILING

Express Mail Label No. EV015808828US
Date of Deposit: March 1, 2002

I hereby certify that this correspondence is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to: BOX PCT, Attn: DO/EO/US, Commissioner for Patents, Washington, DC 20231.

Vickie Diane Prior
Vickie Diane Prior

IN THE UNITED STATES DESIGNATED OFFICE (DO/US)

In re:	Bacher, et al.	Attn: DO/US
Serial No.	To Be Assigned	
Filed:	Concurrently Herewith	
For:	METHOD FOR SCREENING FOR INHIBITORS OF THE BIOSYNTHESIS OF RIBOFLAVIN	

March 1, 2002

Box PCT
Commissioner for Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Sir:

Prior to the examination of the above application, please amend the above-identified application as follows. If any extension of time for the accompanying response or submission is required, Applicant requests that this be considered a petition therefor. The Commissioner is hereby authorized to charge any additional fee, which may be required, or credit any refund, to our Deposit Account No. 50-0220.

In the Specification:

-- RELATED APPLICATIONS

The present application claims priority from International Application No. PCT/EP00/07796, filed on 10 August 2000, which in turn claims priority from German application 199 42 175.7, filed on 3 September 1999, which PCT application was published in English on 15 March 2001, the disclosures of which are hereby incorporated herein by reference in its entirety.--

In the Claims:

Please enter the following amended claims:

3 (amended). The method according to claim 1, wherein said aqueous mixture has a pH in the range of 5.5 to 9.

4 (amended). The method according to claim 1, wherein a premixture is prepared which lacks one essential ingredient and the reaction is started by adding said ingredient.

5 (amended). The method according to claim 1, characterized in that the reaction is terminated by adding an acid or a solvent or a surfactant, preferably trichloroacetic acid or acetone or sodium dodecylsulfate.

6 (amended). The method according to claim 1, characterized in that the level of 6,7-dimethyl-8-ribityllumazine is detected photometrically or fluorometrically.

8 (amended). The method according to claim 1, characterized in that the detection is effected by incubation with riboflavin synthase and detection of riboflavin.

9 (amended). The method according to claim 1, characterized in that a mixture containing 3,4-dihydroxy-2-butanone 4-phosphate is prepared by incubating an aqueous mixture containing ribulose 5-phosphate with 3,4-dihydroxy-2-butanone 4-phosphate synthase.

10 (amended). The method according to claim 1, characterized in that a mixture containing 3,4-dihydroxy-2-butanone 4-phosphate is prepared by incubating an aqueous mixture containing ribose 5-phosphatet with pentose-phosphate isomerase and 3,4-dihydroxy-2-butanone 4-phosphate synthase.

REMARKS

Claims 1-14 are presented for examination. The above claims have been amended to better conform to U.S. practice. Applicants respectfully request substantive examination on the merits. A copy of the claims showing the changes made is attached hereto as a "Version with Markings to Show Changes Made".

Respectfully submitted,

Kenneth D. Sibley
Registration No. 31,665

Myers Bigel Sibley & Sajovec
Post Office Box 37428
Raleigh, North Carolina 27627
Telephone (919) 854-1400
Facsimile (919) 854-1401

CERTIFICATE OF EXPRESS MAILING

"Express Mail" mailing label number EV015808828US
Date of Deposit: March 1, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to Box PCT, Commissioner for Patents, Washington, DC 20231.

Vickie Diane Prior
Date of Signature: March 1, 2002

VERSION WITH MARKINGS TO SHOW CHANGES MADE

3 (amended). The method according to claim**[s]** 1 **[or 2]**, wherein said aqueous mixture has a pH in the range of 5.5 to 9.

4 (amended). The method according to claim**[s]** 1 **[or 2]**, wherein a premixture is prepared which lacks one essential ingredient and the reaction is started by adding said ingredient.

5 (amended). The method according to claim**[s]** 1 **[or 2]**, characterized in that the reaction is terminated by adding an acid or a solvent or a surfactant, preferably trichloroacetic acid or acetone or sodium dodecylsulfate.

6 (amended). The method according to claim**[s]** 1 **[to 5]**, characterized in that the level of 6,7-dimethyl-8-ribityllumazine is detected photometrically or fluorometrically.

8 (amended). The method according to claim**[s]** 1 **[to 6]**, characterized in that the detection is effected by incubation with riboflavin synthase and detection of riboflavin.

9 (amended). The method according to claim**[s]** 1 **[or 2]**, characterized in that a mixture containing 3,4-dihydroxy-2-butanone 4-phosphate is prepared by incubating an aqueous mixture containing ribulose 5-phosphate with 3,4-dihydroxy-2-butanone 4-phosphate synthase.

10 (amended). The method according to claim**[s]** 1 **[or 2]**, characterized in that a mixture containing 3,4-dihydroxy-2-butanone 4-phosphate is prepared by incubating an aqueous mixture containing ribose 5-phosphatet with pentose-phosphate isomerase and 3,4-dihydroxy-2-butanone 4-phosphate synthase.

Method for screening for inhibitors of the biosynthesis of riboflavin

The invention relates to a method for screening for inhibitors of the biosynthesis of riboflavin. It further relates to plant-type enzymes for said method as well as DNA coding for said enzymes. Finally it relates to a method of inhibiting the biosynthesis of riboflavin in plants as well as chemical compounds exhibiting such inhibition.

A promising new approach for finding novel types of herbicides consists in screening libraries of chemical test samples for compounds that inhibit an enzyme in a biochemical pathway that is essential for plants but not for humans or animals. A most promising biosythetic pathway of this type is the pathway of riboflavin biosynthesis. All cellular organisms require riboflavin as an indispensible component of numerous redox enzymes many of which are crucial for the metabolism. All plants generate riboflavin biosynthetically, whereas all animals require a nutritional source of riboflavin. Therefore, an inhibitor for an enzyme in the biosynthesis of riboflavin in plants would not interfere with the metabolism of animals. Furthermore, the absolute amount of riboflavin for cellular activity is low. Therefore, only small amounts of the enzymes for riboflavin biosynthesis are found in cells. This in turm means that only small amounts of an inhibitor for such an enzyme would be-required.

The biosynthetic pathway of riboflavin (Fig. 1) has been studied in considerable detail in bacteria and yeast. The biosynthetic formation of one molecule of riboflavin (7) requires one molecule of GTP and two molecules of ribulose 5-phosphate. GTP (1) is initially converted to the committed product, 2,5-diamino-6-ribosylamino-4(3H)-pyrimidinedione (3) by a sequence of

deamination, side chain reduction, and dephosphorylation. The compound (3) is converted to 6,7-dimethyl-8-ribityllumazine (4) by condensation with 3,4-dihydroxy-2-butanone 4-phosphate (5) wich is obtained enzymatically from ribulose 5-phosphate (6).

It is an object of the invention to provide a method for screening for inhibitors for an enzyme in the plant biosythesis of riboflavin, or for screening for an enzyme that is resistant to a specific inhibitor. It is a further object of the invention to provide a protein having an enzyme activity, useful in such screening method, as well as DNA coding for said protein.

It is further an object of the invention to provide inhibitors as well as a method for inhibiting an enzyme in the biosythesis of riboflavin.

We have discovered that the genomes of plants, specifically *Arabidopsis thaliana*, comprise a gene that codes for a protein that comprises a leader sequence and a sequence exhibiting 6,7-dimethyl-8-ribityllumazine synthase activity. We have expressed this enzyme and found that it is useful for screening a chemical library for inhibitors, with or without the leader sequence.

Specifically we have provided a method for screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityl-lumazine synthase activity comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a plant 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidine dione and 3,4-diyhydroxy-2-butanone 4-phosphate,

(b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the 6,7-dimethyl-8-ribityllumazine,

(e) determining the presence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is lower that the level detected in step (b).

Further we have provided a method for screening for the presence or absence of resistance to inhibition of 6,7-dimethyl-8-ribityllumazine sythase activity comprising the following steps:

(a) preparing a first aqueous mixture containing a protein having a mutated plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidinedione and 3,4-dihydroxy-2-butanone 4-phosphate,

(b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine,

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for 6,7-dimethyl-8-ribityllumazine synthase activity,

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine,

(e) determining the presence of resistance to inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is similar to the level detected in step (b).

Based on the screening method we have thus provided a general solution of the problem of providing inhibitors as well as a method of inhibition for riboflavin biosynthesis in plants.

The invention will now be described in detail.

Identification of the lumazine synthase gene of *Arabidopsis thaliana*
The known amino acid sequence of the ribE protein of *E. coli* (Mörtl et al.) was used to search the DNA sequence data base of the Institute for Genomic Research (Rockville, USA) (accession number, AC004005). Significant similarity was found with a segment of BAC clone F6E13 from *Arabidopsis* chromosome II (Fig.1). The exons of the sequence had been predicted by the computer program xgrail and the predicted protein had been incorrectly assigned as riboflavin synthase.

Sequence comparison showed that the *E. coli* ribE protein was similar over its entire length to the sequence predicted by the *Arabidopsis* gene F6E13.18. However, the putative lumazine synthase gene of *Arabidopsis* also specifies an N-terminal peptide sequence with a high content of serine and threonine (about 67 amino acids) which is devoid of similarity to any sequence in the database and has no equivalent in the *E. coli* protein.

Cloning, expression and purification of lumazine synthase from *Arabidopsis thaliana*

Originator for the construction of an expression vector is cDNA. The *ribE* gene is amplified by PCR with specific primers and cDNA from the corresponding plant as template. Alternatively the cDNA may originate from an existing EST-clone. The RibE protein of *A. thaliana* includes a signal sequence of about 67 amino acids which was found not to be essential for enzyme activity.

The amplified DNA fragment is modified by two consecutive PCR amplifications with modifying primers. In the first PCR reaction a ribosomal binding site preceding the start codon at an optimal distance is introduced at the 5´-end. A recognition site for a restriction enzyme, for example BamHI, SalI or PstI is introduced at the 3´-end. The preferred recognition site is BamHI. In the second PCR reaction the product of the first is used as template. At the 5´-end a recognition site for the restriction enzyme EcoRI preceding the ribosomal binding site is introduced with a modifying primer. The amplified DNA fragment is inserted into a vector capable of autonomous replication in the host microorganism to give a recombinant plasmid containing said DNA. The recombinant plasmid is used to transform the host microorganism, for example *Escherichia coli* or *Bacillus subtilis*. The preferred host is *E. coli*. The expression of plant protein may be poor in the host organism. To enhance the expression level and/or to simplify the purification of the protein the recombinant plasmid may include a gene or a part of a gene without a stop codon preceding the *ribE* gene in the same reading frame. A preferred gene for this purpose is the *malE* gene from *E. coli*. The expression of such a recombinant DNA generates a fusion protein between the maltose binding protein (MBP) from *E. coli* and the plant RibE protein. Various constructs are shown in Fig. 3. Fig. 3A shows a construct with putative signal sequence S, Fig. 3B shows a construct without the putative signal sequence. Fig. 3C shows a construct with maltose binding protein and without putative signal sequence.

The strains harbouring the expression vectors can be cultivated in conventional culture media at 15 to 40°C. The preferred temperature is 37°C. The *E. coli* strains are induced with 0.5 to 2 mM isopropyl-β-D-thiogalactosid at an optical density from 0.5 to 0.8. The cells are incubated between 2 and 12 h, preferably 5 h. The cells are lysed with lysozyme and/or disrupted with a sonifier. The crude extract with MBP-ribE fusion protein is purified by affinity chromatography with an amylose resin. A protein is obtained which has the proper folding

structure for exhibiting the desired enzyme activity.

Screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase

Lumazine synthase catalyzes the formation of 6,7-dimethyl-8-ribityllumazine by condensation of 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidinedione with 3,4-dihydroxy-2-butanon 4-phosphate (Neuberger et al., 1986; Volk & Bacher, 1988). The enzyme requires no cofactors and shows full catalytic activity in the presence of a chelator such as EDTA. 3,4-dihydroxy-2-butanon 4-phosphate is prepared enzymatically from ribulose 5-phosphate by the action of 3,4-dihydroxy-2-butanon 4-phosphate synthase (Richter et al., 1992). The assay can be started by adding one of the needed substances to a mixture of the others. Preferably 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidinedione is added to a solution of 3,4-dihydroxy-2-butanon 4-phosphate and enzyme in a buffer at pH 6.5 to 8.0, preferably 7.0. The reaction mixture can be incubated for 1 to 60 min at 10 to 40°C. Preferably it is incubated for 5 min at 37°C. The assay can be stopped by denaturing the enzyme with trichloroacetic acid, acetone or sodium dodecylsulfate. The preferred denaturing is with trichloroacetic acid. The assay is carried out with otherwise identical mixtures with and without test sample of a possible inhibitor. The enzyme product 6,7-dimethyl-8-ribityllumazine can be detected directly without derivatization, preferably photometrically, preferentially after purification by HPLC. The lumazine can be identified by absorbance at 410 nm. The extinction coefficient is 10300 $M^{-1}cm^{-1}$. The product can also be monitored fluorometrically at an absorbance wavelength at 408 nm and an emision wavelength at 487 nm. 6,7-Dimethyl-8-ribityllumazine can also be synthesized without enzymatic catalysis (Bacher et al., 1996). For an exact determination of the enzymatic activity it is therefore necessary to subtract the amount of lumazine formed non-catalytically from the entire amount of produced lumazine.

The isolated DNA codes specifically for a protein with a plant-type sequence of 6,7-dimethyl-8-ribityllumazine synthase whereby it may have either a single open reading frame for said protein or additionally at least one further open reading frame coding for another enzyme of the flavin pathyways.

The term "plant-type sequence" means in one sense a sequence as it occurs in a plant (with or without leader sequence). In a broader and more adequate sense, it means a sequence of a sequence space which is established by using a specific plant 6,7-dimethyl-8-ribityllumazine synthase (with or without leader sequence) as a reference sequence, producing an alignment of said reference sequence with at least one other plant 6,7-dimethyl-8-ribityllumazine synthase sequence and obtaining for each position in said alignment a set of equivalent amino acids from the variability at this position. Any sequence contained in this sequence space has a very high likelihood of being functional for the intended enzyme activity and being highly homologous to plant enzymes.

It is surprising that the isolated proteins could be obtained in a form (e.g. folding form) which is functional for the intended enzyme activity. This functional competence defines a subset of substances of the much broader set of substances which all have the same sequence.

For the screening process, a specific plant enzyme may be used selected in accordance with the plant(s) targeted. It is also possible to construct a plant-type enzyme sequence (with or without leader sequence) which is on average closest to each of a subset of plant enzyme sequences of a set of plants targeted.

Example 1: Construction of an expression clone

The putative *ribE* gene (gene F6E13.18) of E. coli was amplified by PCR using a cDNA library (Minet et al.) as template
First PCR:

The reaction mixtures contained 10 pmol primer 5´-GGAGAAATTAACCA TGAAGT-CATTAGCTTCGCCG-3´, 10 pmol primer 5´-TCATGTGGATCCA TGGAACGAGCCGAG-3´, 10 ng of cDNA, 1μl Taq polymerase, 10 μl of buffer (Eurogentec), 6 μl $MgCl_2$ (25 mM, Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μl.

The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 95°C, 45 sec at 50°C, 45 sec at 72°C) were performed. After another 7 min incubation at 72°C, the mixture was cooled at 4 °C, and the DNA was electrophoresed on a 0.8 % agarose gel. The band at 750 bp was purified with a gel extraction kit (Qiagen). The DNA fragment was excised from the agarose gel with a scalpel. Three volumes of buffer QX1 (Qiagen) were added to 1 volume of the excised gel and incubated at 50°C for 10 min. One gel volume of isopropanol was added. To bind DNA, the sample was applied to a Qiaquick column and centrifuged for 1 min at 14000 rpm. The flow through was discarded. 0.75 ml buffer PE (Qiagen) were added to the column and centrifuged as before. The flow through was discarded and the column was centrifuged for an additional 1 min at 14000 rpm. The column was placed in a clean 1.5 ml Eppendorf tube. 50 μl of H_2O (bidestilled, sterile) were added to the column and it was centrifuged for 1 min at 14000 rpm. The flow through contained the purified DNA.

Second PCR (Two identical PCRs with 100 μl each were performed to obtain a

higher yield)

10 pmol primer CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG-3´, 10 pmol 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 1 µl of Taq polymerase (1 U), 10 µl of buffer (Taq-buffer, Eurogentec), 6 µl $MgCl_2$ (25 mM), 5 µl of purified PCR1 product and 20 nmol dNTPs in a total volume of 100 µl.
The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 94 °C, 45 sec at 50°C, 45 sec at 72 °C) were performed. After another 7 min incubation at 72°C, the mixture was cooled at 4°C, and the DNA was purified with a PCR purification kit (Qiagen).

5 volumes of buffer PB (Qiagen) were added to 1 volume of the PCR reaction, applied to a Qiaquick column and centrifuged for 1 min at 14000 rpm. The flow through was discarded. 0.75 ml buffer PE (Qiagen) were added to the column and centrifuged as before. Plasmid pNCO113 (Stüber et al., 1980) was isolated from 20 ml overnight culture of pNCO113 in *E. coli* XL-1 Blue using the plasmid isolation kit Nucleobond AX100 (Macherey & Nagel).

The PCR product and the plasmid pNCO113 were digested with the restriction enzymes EcoRI and BamHI, 20 µl OPA buffer (Pharmacia), 2 µl EcoRI (20 U, Pharmacia), 2 µl BamHI (20 U, Pharmacia), 2,5 µg PCR2 product resp. 5.0 µg pNCO113 in a total of 100 µl H_2O) at 37°C for 4 h and purified with a PCR purification kit. The digested PCR2 product and the plasmid pNCO113 were ligated together with T_4-ligase yielding plasmid pNCOribE(AT): 50 fmol of pNCO113, 100 fmol of PCR2 product, 4 µl of buffer (Gibco), and 1 µl of T_4-ligase (1 U, Gibco) in a total of 20 µl. The mixture was incubated overnight at 4°C, purified with a PCR purification kit and transformed into electrocompetent *E. coli* XL-1 Blue cells (Bullock et al., 1987) by electroporation.

Preparation of the electrocompetent cells: One liter of Luria-Bertani-medium was

inoculated with 10 ml of a fresh overnight culture. The cells were grown at 37 °C with vigorous shaking to an optical density of 0.5 to 0.7. The suspension was chilled on ice for 20 min and centrifuged in a cold rotor at 4000 g for 15 min at 4°C. The supernatant was removed and the pellet resuspended in 1 liter of ice-cold sterile 10 % glycerol. The cells were pelleted two times as described before and the pellet was resuspended the first time in 0.5 liter and the second time in 20 ml of ice-cold 10 % glycerol. The cells were again pelleted, and the pellet was resuspended to a final volume of 2 to 3 ml in ice-cold 10 % glycerol. The suspension was frozen in aliquots of 80 µl and stored in liquid nitrogen.

Electro-transformation using the Gene Pulser apparatus from Biorad: The electrocompetent cells were thawed at room temperature and placed on ice. 40 µl of the cell suspension were mixed with 1 µl of the ligation mixture and transferred into a sterile 0.2 cm cuvette (Biorad). The suspension was shaked to the bottom and the cuvette was placed into the chamber slide. The chamber slide was pushed into the chamber and a pulse was applied (2,50 kV, 25 µF, Pulse Controller 200 Ohm). The cuvette was removed from the chamber and the cells were suspended in 1 ml Soc medium (2 % casein hydrolysate, 0.5 % yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). The suspension was incubated with shaking for 1 h at 37°C and plated on LB media supplemented with 150 mg ampicillin per liter. Plasmids from different clones were isolated (pNCOribE(AT)1-10).

The plasmids were isolated from 5 ml of fresh overnight culture using the mini plasmid isolation kit from Qiagen. The bacterial pellet was resuspended in 0.3 ml of 50 mM Tris hydrochloride, pH 8.0 containing 10 mM EDTA and 100 µg/ml RNase. 0.3 ml of 200 mM sodium hydroxide containing 1 % SDS were added, and the mixture was incubated for 5 min at room temperature. 0.3 ml of chilled 3.0 M sodium acetate, pH 5.5 were added, and the mixture was incubated on ice for 10 min. The mixture was centrifuged for 15 min at 14000 rpm in a

minifuge. The supernatant was removed and applied to a Qiagen-tip 20 which was previously equilibrated with 1 ml of 50 mM MOPS , pH 7.0, containing 750 mM NaCl, 15 % ethanol and 0.15 % Triton X-100. The Qiagen tip was washed 4 times with 1 ml of 50 mM MOPS, pH 7.0 containing 1000 mM NaCl and 1 % ethanol. The DNA was eluted with 0.8 ml of 50 mM Tris hydrochloride, pH 8.5 containing 1250 mM NaCl and 15 % ethanol. The DNA was precipitated with 0.7 volumes of isopropanol, centrifuged at 14000 rpm for 30 min and washed with 1 ml cold 70 % ethanol. The DNA sequence of the recombinant plasmids were determined by a automated dideoxynucleotide sequencing methode using an ABI Prism 377 DNA sequencer from Applied Biosystems Inc. with the ABI Prism Sequencing Analysis Software.

Example 2: Construction of an expression clone without the putative transit peptide sequence

The *ribE* gene with the exception of the first 216 bp coding for a putative transit peptide was amplified by PCR using plasmid pNCOribE(AT)1 (from *A. thaliana* ribE expression clone) as template. Plasmid pNCOribE(AT)1 was isolated as described. Amino acid 73 was mutated from an arginine to methionine and a recognition site for the restriction enzyme EcoRI preceding the start codon was introduced at the 5´ end with a modifying primer.

First PCR:
The reaction mixtures contained 10 pmol primer 5´-GGAGAAATTAACCATGCATGTTA-CGGGGTCTCTTATC-3´, 10 pmol primer 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 10 ng of cDNA, 1 μl Taq polymerase (1 U), 10 μl of buffer (Eurogentec), 6 μl of $MgCl_2$ (25 mM, Eurogentec) and 20 nmol of dNTPs in a total volume of 100 μl.
The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 94°C, 45 sec at 50°C, 45 sec at 72°C) were performed. After another 7 min

incubation at 72°C, the mixture was cooled at 4°C, and the DNA was electrophoresed on a 0.8 % agarose gel. The band at 500 bp was purified with a gel extraction kit (Qiagen).

Second PCR (Two identical PCRs with 100 μl each were performed to obtain a higher yield)

10 pmol primer CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG-3´, 10 pmol 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 1 μl of Taq polymerase (1 U), 10 μl of buffer (Taq-buffer, Eurogentec), 6 μl of $MgCl_2$ (25 mM), 5 μl of purified PCR1 product and 20 nmol dNTPs in a total volume of 100 μl.
The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 94°C, 45 sec at 50°C, 45 sec at 72°C) were performed. After another 7 min incubation at 72°C, the mixture was cooled at 4°C, and the DNA was purified with a PCR purification kit (Qiagen).

The further steps were analogous to the construction of pNCOribE(AT) in Reference Example 1. The resulting plasmid encoding a ribE protein without the putative peptide sequence is designated pNCOribE(AT)-sig.

Example 3: Construction of an malE-ribE fusion clone without putative transit peptide sequence

The *ribE* gene with the exception of the first 216 bp was amplified by PCR using plasmid pNCOribE(AT) as template and was ligated in frame at the 3´-end of the *malE* gene.
First PCR:
The reaction mixtures contained 10 pmol primer 5´-ATAATAATAGCGGCCGCTATGCATGT-TACGGGGTCTCTTATC -3´, 10 pmol primer 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 10 ng of cDNA, 1μl Taq

polymerase (1 U), 10 µl of buffer (Eurogentec), 6 µl $MgCl_2$ (25 mM, Eurogentec) and 20 nmol of dNTPs in a total volume of 100 µl.
The mixture was denatured at 94°C for 5 min. 30 PCR cycles (60 sec at 94° C, 45 sec at 50° C, 45 sec at 72°C) were performed. After another 7 min incubation at 72° C, the mixture was cooled at 4° C, and the DNA was electrophoresed on a 0.8 % agarose gel. The band at 500 bp was purified with a gel extraction kit (Qiagen).

Second PCR (Two identical PCRs with 100 µl each were performed to obtain a higher yield)
10 pmol primer CAATTTGAATTCATTAAAGAGGAGAAATTAACTATG-3´, 10 pmol 5´-TCATGTGGATCCATGGAACGAGCCGAG-3´, 1 µl of Taq polymerase (1 U), 10 µl of buffer (Taq-buffer, Eurogentec), 6 µl $MgCl_2$ (25 mM), 5 µl of purified PCR1 product and 20 nmol dNTPs in a total volume of 100 µl.
The mixture was denatured at 94° C for 5 min. 30 PCR cycles (60 sec at 94° C, 45 sec at 50° C, 45 sec at 72°C) were performed. After another 7 min incubation at 72° C, the mixture was cooled at 4° C, and the DNA was purified with a PCR purification kit (Qiagen).

Plasmid pNCOmalEribH (Fischer, 1997) was isolated from 20 ml of a overnight culture as described for plasmid pNCO113.
The PCR product and the plasmid pNCOmalEribH were digested with the restriction enzymes NotI and BamHI (10 µl BamHI buffer (NEB), 1 µl BSA (100x), 4 µl BamHI (20 U, NEB), 3 µl NotI (60 U, NEB), 2 µg PCR product resp. 5 µg pNCOmalEribH in a total volume of 100 µl) at 37° C for 3 h. The PCR product was purified with a PCR purification kit (Qiagen). The digested plasmid was electrophoresed on an agarose gel. The band at 3400 bp was purified with a gel extraction kit (Qiagen). Further steps were analogous to the construction of pNCOribE(AT) in Example 1. the plasmid encoding a malE-ribE fusion protein was named pNCOmalE-ribE(AT)-sig

Example 4: Preparation and purification of the ribE proteins with and without signal sequence

1 l Luria Bertani (LB) medium containing 160 mg ampicillin were inoculated with 40 ml overnight culture of *E. coli* strain XL-1 harboring plasmid pNCOribE(AT) resp. pNCOribE(AT)-sig. The culture was grown in shaking culture at 37°C. At an optical density (600 nm) of 0.6 the culture was induced with 2mM IPTG. The culture was grown for another 5 h. The cells were harvested by centrifugation for 20 min at 5000 rpm and 4°C. The cells were washed with 0.9 % NaCl solution, centrifugated as above and frozen at -20° C for storage. The cells were thawed in 20 ml 50 mM potassiumphosphate pH 7.0 containing 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. The mixture was sonified 6 x 15 sec (Branson sonifier level 4). The suspension was centrifuged at 15000 rpm at 4°C for 20 min. The supernatant was applied to a 20 ml column of Sepharose Q (Pharmacia) previously equillibrated with 50 mM potassium phosphate pH 7.0 (buffer A). the column was washed with 60 ml buffer A and developed with a linear gradient of 200 ml buffer A containing 1 M NaCl. Fractions containing the ribE protein were identified by SDS electrophorese and concentrated with a Amicon cell (membran size, 30 kDa). Aliquots containing 5 mg protein were passed through a Superdex 200 (1.6 by 60 cm, Pharmacia) gel filtration column , which was developed with buffer A containing 100 mM NaCl. 38 mg ribE(AT) resp. 2 mg ribE(AT)-sig were obtained.

Example 5: Preparation and purification of the MBP-ribE fusion proteins

0.5 l Luria Bertani (LB) medium containing 75 mg ampicillin were inoculated with 40 ml overnight culture of *E. coli* strain XL-1 harboring plasmid pNCOmalEribE(AT). The culture was grown in shaking culture at 37°C. At an optical density (600 nm) of 0.5 the culture was induced with 1mM IPTG. The

culture was incubated with shaking for another 3 h. The cells were harvested by centrifugation for 20 min at 5000 rpm and 4°C. The cells were washed with 0.9 % NaCl solution, centrifuged as above and stored at -20°C. The cells were thawed in 10 ml 50 mM potassium phosphate, pH 7.0 containing 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride. The mixture was sonified 6 x 15 sec (Branson sonifier level 4). The suspension was centrifuged at 15000 rpm at 4°C for 20 min. The supernant was diluted 1:5 with buffer A (Example 4) and placed on a 2 ml column of amylose resin (New England Biolabs) previously equilibrated with 15 ml buffer A. The column was washed with 30 ml buffer A. The fusion protein was eluted from the column with 6 ml buffer A containing 10 mM maltose. 5 mg malE-ribE protein was obtained. The purity of the protein (56 kDa) was examined by SDS electrophorese.

Example 6: Screening for lumazine synthase activity

Preparation of 3,4-dihydroxy-2-butanon-4-phosphate:
A reaction mixture contained 100 mM potassium phosphate pH 7.5, 20 mM $MgCl_2$, 10 mM ribose 5-phosphate (Sigma) and 0.1 U pentose-phosphate isomerase (Sigma) and 3,4-dihydroxy-2-butanon 4-phosphate synthase (2000 U) in a total volume of 100 μl. The mixture was incubated at 37°C for 20 min.

Assay of lumazine synthase activity:
Assay mixture contained 100 mM potassium phosphate, pH 7.0, 20 mM EDTA, 1 mM 3,4-dihydroxy-2-butanone 4-phosphate, and 5 μl of the enzyme sample in a total volume of 50 μl. After a preincubation time of 2 min at 37°C the reaction was started by the addition of 1 μl of 5 mM 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimidinedione and the samples were incubated at 37°C for 5 min. The reaction was stopped by the addition of 50 ml trichloroacetic acid (15 %). 6,7-Dimethyl-8-ribityllumazine was determined by reverse-phase HPLC on a column of Nucleosil RP18. The eluent contained 30 mM formic acid and 20 %

methanol. The effluent was monitored fluorometrically (excitation, 408 nm; emission, 487 nm). One unit of enzyme activity catalyzes the formation of 1 nmol of 6,7-dimethyl-8-ribityllumazine per h at 37°C.

construct	specific activity (nmol h-1 mg-1)	
	cell extract	purified protein
E. coli XL-1 (host strain)	4	----
pNCOribE(AT)	48	8740
pNCOribE(AT)-sig	835	12000
pNCOmalE-ribE(AT)-sig	203	9130

Screening for inhibition of lumazine synthase activity

The screening for inhibition of lumazine synthase was done with the ribE gene product without the putative transit peptide sequence. The assays were performed with different inhibitor concentrations at constant concentrations of 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidindione, and 3,4-dihydroxy-2-butanone 4-phosphate. The amount of biomimetically formed lumazine was substracted from the total amount of lumazine.

Screening for lumazine synthase activity in the presence of 5-nitroso-6-ribitylamino-2,4(1H,3H)pyrimidinedione (Winestock & Plaut, 1961) or 5-nitro-6-ribitylamino-2,4(1H,3H)pyrimidinedione (Cresswell & Wood, 1960)

test compound	5-nitro-6-ribitylamino-2,4(1H,3H)pyrimidinedione		5-nitroso-6-ribitylamino-2,4(1H,3H)pyrimidinedione	
concentration [μM]	lumazine formation per min [μM/min]	enzymatic activity [nmol/h mg]	lumazine formation per min [μM/min]	enzymatic activity [nmol/h mg]
0	26,41	9080 (100%)	27,09	9314 (100%)
5	26,41	9080 (100%)	26,21	9011 (97%)
10	25,73	8730 (96%)	25,14	8643 (93%)
100	20,00	6980 (77%)	23,40	8045 (86%)
1000	6,89	2070 (23%)	9,61	3304 (35%)
5000	3,01	980 (11%)	1,46	502 (5%)
10000	2,62	650 (7%)	1,07	368 (4%)
biomimetically formed lumazine	0,1		0,1	

Compound	IC_{50} [μM]
5-Nitro-6-ribitylamino-2,4(1H,3H)pyrimidinedione	310
5-Nitroso-6-ribitylamino-2,4(1H,3H)pyrimidinedione	560

500 μl potassium phosphate buffer (100 mM, pH 7.5) containing 1 mM EDTA and 10 μg enzyme, 100 μl inhibitor (final concentration, 0 - 10 mM), and 10 μl 5-amino-6-ribitylamino-2,4(1H,3H)-pyrimdinedione (9 mM) were combined in a 1 ml cuvette. After a preincubation time of 10 min at 37°C, the reaction was started by the addition of 20 μl 3,4-dihydroxy-2-butanone 4-phosphate and incubated for another 5 min at 37°C. The reaction was monitored photometrically at 410 nm.

Literature

Bullock W.O., J.M. Fernandez, J.M. Short (1987)
XL-1 Blue: a high efficiency transforming recA Escherichia coli strain with β-Galactosidase selection
BioTechiques, 5, 376-379

Cresswell R.M and Wood H.C.S (1960)
The biosynthesis of pteridines. Part I. the synthesis of riboflavin
J. Chem. Soc., 1960, 4769-4775

Fischer M. (1997)
Effiziente Methoden zur in vitro Mutagenese: Untersuchungen zum Mechanismus von Lumazinsynthase und GTP-Cyclohydrolase I
Thesis, Technische Universität München

Mörtl S., M. Fischer, G. Richter, J. Tack, S. Weinkauf and A. Bacher (1996)
Biosynthesis of Riboflavin: Lumazine synthase of Escherichia coli
J. Biol. Chem., 271, 33201-33207

Neuberger G. and A. Bacher (1986)
Biosynthesis of ribofalvin. Enzymatic formation of 6,7-dimethyl-8-ribityllumazine by riboflavin synthase from Bacillus subtilis
Biochem. Biophys.Res. Commun., 139, 1111-1116

Richter G., R. Volk, C. Krieger, H. Lahm, U. Röthlisberger and A. Bacher (1992)
Biosynthesis of riboflavin. Cloning, sequencing and expression of the gene coding for 3,4-dihydroxy-2-butanon-4-phosphate synthase of Escherichia coli
J. Bacteriol., 174, 4050-4056

Stüber D., H. Matile, G. Garotta (1990)
System for high-level production in Escherichia coli and rapid purification of recombinant proteins: Application to epitope mapping, preparation of antibodies and structure-function analysis
in: Lefkovitz I., P. Pernis (eds.) Immunological Methods, IV, 121-152

Volk R. and A. Bacher (1988)
Biosynthesis of riboflavin. The structure of the 4-carbon precursor
J. Amer. Soc, 110, 3651-3653

Winestock C.H. and Plaut G.W.E. (1961)
Synthesis and properties of certain substituted lumazines
J. Org. Chem., 26, 4456-4462

Annex A

Nucleotide- and amino acid sequence of the lumazine synthase of *Arabidopsis thaliana*

```
ATGAAGTCATTAGCTTCGCCGCCGTGTCTCCGCCTGATACCGACGGCACACCGTCAGCTC
 M  K  S  L  A  S  P  P  C  L  R  L  I  P  T  A  H  R  Q  L

AATTCGCGTCAATCTTCCTCCGCCTGTTATATACACGGTGGCTCTTCTGTGAACAAATCC
 N  S  R  Q  S  S  S  A  C  Y  I  H  G  G  S  S  V  N  K  S

AATAATCTCTCATTCTCCTCATCCACATCCGGATTTGCGTCACCACTAGCTGTAGAGAAG
 N  N  L  S  F  S  S  S  T  S  G  F  A  S  P  L  A  V  E  K

GAATTACGCTCTTCATTCGTACAGACGGCTGCTGTTCGCCATGTTACGGGGTCTCTTATC
 E  L  R  S  S  F  V  Q  T  A  A  V  R  H  V  T  G  S  L  I

AGAGGCGAAGGTCTTAGATTCGCCATCGTGGTAGCTCGTTTCAATGAGGTTGTGACTAAG
 R  G  E  G  L  R  F  A  I  V  V  A  R  F  N  E  V  V  T  K

TTGCTTTTGGAAGGAGCGATTGAGACTTTCAAGAAGTATTCAGTCAGAGAAGAAGACATT
 L  L  L  E  G  A  I  E  T  F  K  K  Y  S  V  R  E  E  D  I

GAAGTTATTTGGGTTCCTGGCAGCTTTGAAATTGGTGTTGTTGCACAAAATCTTGGGAAA
 E  V  I  W  V  P  G  S  F  E  I  G  V  V  A  Q  N  L  G  K

TCGGGAAAATTTCATGCTGTTTTATGTATCGGCGCTGTGATAAGAGGAGATACCACACAT
 S  G  K  F  H  A  V  L  C  I  G  A  V  I  R  G  D  T  T  H

TATGATGCTGTTGCCAACTCTGCTGCGTCTGGAGTACTTTCTGCTAGCATAAATTCAGGC
 Y  D  A  V  A  N  S  A  A  S  G  V  L  S  A  S  I  N  S  G

GTTCCATGCATATTTGGTGTACTGACTTGCGAGGACATGGATCAGGCTCTGAATCGATCT
 V  P  C  I  F  G  V  L  T  C  E  D  M  D  Q  A  L  N  R  S

GGTGGCAAAGCCGGCAATAAGGGAGCTGAAACTGCTTTGACGGCGCTCGAAATGGCGTCG
 G  G  K  A  G  N  K  G  A  E  T  A  L  T  A  L  E  M  A  S

TTGTTTGAGCACCACCTGAAATAGCTCGGCTCGTTCCAT
 L  F  E  H  H  L  K
```

Claims

1. A method for screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase activity comprising the following steps:
   (a) preparing a first aqueous mixture containing a protein having a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidine-dione and 3,4-dihydroxy-2-butanone 4-phosphate;
   (b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine;
   (c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample;
   (d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine;
   (e) determining the presence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is lower than the level detected in step (b).

2. A method for screening for the presence or absence of resistance to inhibition of 6,7-dimethyl-8-ribityllumazine synthase activity comprising the following steps:
   (a) preparing a first aqueous mixture containing a protein having a mutated plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribityl amino-2,4(1H,3H)pyrimidione and 3,4-dihydroxy-2-butanone 4-phosphate;
   (b) reacting said first mixture during a predetermined period of time at

a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine;

(c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a specific inhibitor for 6,7-dimethyl-8-ribityllumazine synthase activity;

(d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine;

(e) determining the presence of resistance to inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is similar to the level detected in step (b).

3. The method according to claims 1 or 2, wherein said aqueous mixture has a pH in the range of 5.5 to 9.

4. The method according to claims 1 or 2, wherein a premixture is prepared which lacks one essential ingredient and the reaction is started by adding said ingredient.

5. The method according to claims 1 or 2, characterized in that the reaction is terminated by adding an acid or a solvent or a surfactant, preferably trichloroacetic acid or acetone or sodium dodecylsulfate.

6. The method according to one of the claims 1 to 5, characterized in that the level of 6,7-dimethyl-8-ribityllumazine is detected photometrically or fluorometrically.

7. The method according to claim 6, wherein the detection is effected in an HPLC fraction.

8. The method according to one of claims 1 to 6, characterized in that the detection is effected by incubation with riboflavin synthase and detection of riboflavin.

9. The method according to one of claims 1 or 2, characterized in that a mixture containing 3,4-dihydroxy-2-butanone 4-phosphate is prepared by incubating an aqueous mixture containing ribulose 5-phosphate with 3,4-dihydroxy-2-butanone 4-phosphate synthase.

10. The method according to one of claims 1 or 2, characterized in that a mixture containing 3,4-dihydroxy-2-butanone 4-phosphate is prepared by incubating an aqueous mixture containing ribose 5-phosphate with pentose-phosphate isomerase and 3,4-dihydroxy-2-butanone 4-phosphate synthase.

11. An isolated protein having a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence and existing in a form functional for 6,7-dimethyl-8-ribityllumazine synthase activity.

12. An isolated DNA coding exclusively for
    (a) a protein comprising a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence; and
    (b) optionally at least one additional enzyme of the flavin biosynthetic pathways.

13. A method of inhibiting an enzyme with 6,7-dimethyl-8-ribityllumazine synthase activity of or in a plant by treatment with a compound selected from the group of chemical compounds that exhibit inhibition in the screening method of claim 1.

14. A chemical compound exhibiting inhibition of a plant 6,7-dimethyl-8-ribityllumazine synthase activity in the method of claim 1.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
15 March 2001 (15.03.2001)

PCT

(10) International Publication Number
WO 01/18233 A2

(51) International Patent Classification[7]: C12Q 1/00

(21) International Application Number: PCT/EP00/07796

(22) International Filing Date: 10 August 2000 (10.08.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
199 42 175.7 3 September 1999 (03.09.1999) DE

(71) Applicant and
(72) Inventor: **BACHER, Adelbert** [DE/DE]; Königsbergerstr. 74, 85748 München (DE).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **MÖRTL, Simone** [DE/DE]; Hilgertshausenerstr. 851, 85305 Volkersdorf (DE).

(74) Agents: **HARTZ, Nikolai** et al.; Wächtershäuser & Hartz, Tal 29, 80331 München (DE).

(81) Designated States *(national)*: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *Without international search report and to be republished upon receipt of that report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/18233 A2

(54) Title: METHOD FOR SCREENING FOR INHIBITORS OF THE BIOSYNTHESIS OF RIBOFLAVIN

(57) Abstract: A method is described for screening for the presence or absence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase activity comprising the following steps : (a) preparing a first aqueous mixture containing a protein having a plant-type 6,7-dimethyl-8-ribityllumazine synthase sequence, 5-amino-6-ribitylamino-2,4(1H,3H)pyrimidine-dione and 3,4-dihydroxy-2-butanone 4-phosphate; (b) reacting said first mixture during a predetermined period of time at a predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine; (c) preparing a second aqueous mixture by including in said first mixture a predetermined amount of a chemical test sample; (d) reacting said second mixture during the predetermined period of time at the predetermined temperature and subsequently detecting the level of 6,7-dimethyl-8-ribityllumazine; (e) determining the presence of inhibition of 6,7-dimethyl-8-ribityllumazine synthase by observation of whether the level detected in step (d) is lower than the level detected in step (b).

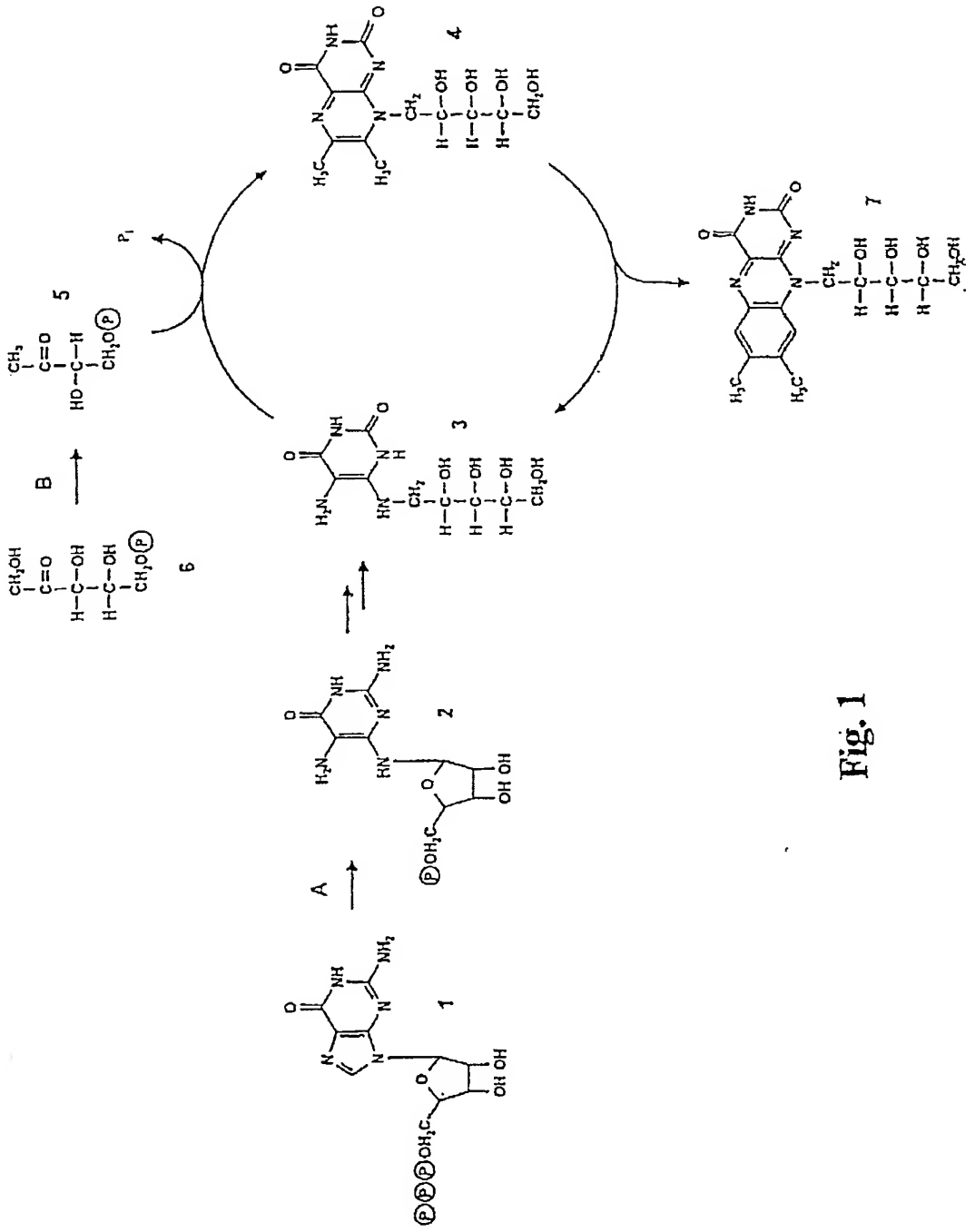
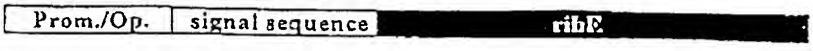


Fig. 1

```
1.  MKSLASPPCLRLIPTAHRQLNSRQSSSACYIHGGSSVNKSNNLSFSSST
2.
3.

1.  SGFASPLAVEKELRSSFVQTAAVRHVTGSLIRGEGLRFAIVVARFNEVV
2.                              MNIIEANVATPDARVAITIARFNNFI
3.                              MNIIQGNLVGTGLKIGIVVGRFNDFI

1.  TKLLLEGAIETFKKYS-VREEDIEVIWVPGSFEIGVVAQNLGKSGKFHA
2.  NDSLLEGAIDALKRIGQVKDENITVVWVPGAYELPLAAGALAKTGKYDA
3.  TSKLLSGAEDALLRHG-VDTNDIDVAWVPGAFEIPFAAKKMAETKKYDA

1.  VLCIGAVIRGDTTHYDAVANSAASGVLSASINSGVPCIFGVLTCEDMDQ
2.  VIALGTVIRGGTAHFEYVAGGASNGLAHVAQDSEIPVAFGVLTTESIEQ
3.  IITLGTVIRGATTHYDYVCNEAAKGIAQAANTTGVPVIFGIVTTENIEQ

1.  ALNRSGGKAGNKGAETALTALEMASLFEHHLK     A.thaliana
2.  AIERAGTKAGNKGAEAALTALEMINVLKAIKA     E.coli
3.  AIERAGTKAGNKGVDCAVSAIEMANLNRSFE      B.subtilis
```

Fig. 2

(A) pNCO-sig-ribE

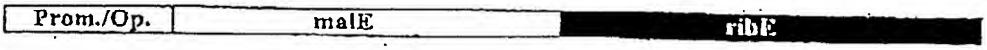


transit signal peptide lumazine synthase

(B) pNCO-ribE

lumazine synthase

(C) pNCO-malE-ribE



maltose binding protein lumazine synthase

Fig. 3

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

Attorney Docket No. 9286-9

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled **METHOD FOR SCREENING OF INHIBITORS OF THE BIOSYNTHESIS OF RIBOFLAVIN,**

the specification of which

☐ is attached hereto

OR

☒ was filed on March 1, 2002 as United States Application No. 10/080,144 or PCT International Application Number PCT/EP00/07796 and was amended on _____ (if applicable).

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to patentability as defined in Title 37 Code of Federal Regulations, §1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT International application which designated at least one country other than the United States of America, listed below and have also identified below any foreign application for patent or inventor's certificate, or of any PCT International application having a filing date before that of the application on which priority is claimed.

19942175.7	Germany	09/03/1999	☒ Yes ☐ No
Number	Country	MM/DD/YYYY Filed	Priority Claimed
			☐ Yes ☐ No
Number	Country	MM/DD/YYYY Filed	Priority Claimed
			☐ Yes ☐ No
Number	Country	MM/DD/YYYY Filed	Priority Claimed

I hereby claim the benefit under Title 35, United States Code, § 119(e) of any United States provisional application(s) listed below.

Application Number(s)	Filing Date (MM/DD/YYYY)
Application Number(s)	Filing Date (MM/DD/YYYY)

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) or § 365(c) of any PCT international application designating the United States of America, listed below.

PCT/EP00/07796	10/10/2000	Published
Appln. Serial No.	Filing Date	Status Patented/Pending/Abandoned
Appln. Serial No.	Filing Date	Status Patented/Pending/Abandoned
Appln. Serial No.	Filing Date	Status Patented/Pending/Abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true: and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following registered attorney(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. I also appoint the following registered attorney(s) to represent me before all competent International Authorities in connection with any and all international applications filed by me with an appropriate receiving office claiming priority to the U.S. application. I also appoint the following registered attorney(s) to make or receive payment on my behalf in connection with the filing of such international applications.

Customer Number:

20792
PATENT TRADEMARK OFFICE

Send correspondence to:

Customer Number:

20792
PATENT TRADEMARK OFFICE

Direct telephone calls to: Kenneth D. [illegible]
(919) 854-1400

Facsimile: (919) 854-1401

Full name of first inventor: **Adelbert Bacher**

Inventor's Signature: A. Bacher Date: 13. 6. 2002

Residence: Garching, Germany DE

Citizenship: Germany

Mailing Address: Königsbergerstr. 74
D-85748 Garching, Germany

Full name of second inventor: **Simone Mörtl**

Inventor's Signature: S. Mörtl Date: 20. 6. 2002

Residence: Volkersdorf, Germany DE

Citizenship: Germany

Mailing Address: Hilgertshausenerstr. 851
D-85305 Volkersdorf, Germany

SEQUENCE LISTING

<110> Bacher, Adelbert

<120> Method for screening for inhibitors of the biosynthesis of riboflavin

<130> PCT-11222

<140>
<141>

<150> DE 199 42 175.7
<151> 1999-09-03

<160> 12

<170> PatentIn Ver. 2.1

<210> 1
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 1
ggagaaatta accatgaagt cattagcttc gccg 34

<210> 2
<211> 27
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 2
tcatgtggat ccatggaacg agccgag 27

<210> 3
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 3
caatttgaat tcattaaaga ggagaaatta actatg 36

<210> 4
<211> 37
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 4
ggagaaatta accatgcatg ttacggggtc tcttatc 37

<210> 5
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Primer

<400> 5
ataataatag cggccgctat gcatgttacg gggtctctta tc 42

<210> 6
<211> 699
<212> DNA
<213> Arabidopsis thaliana

<220>
<221> CDS
<222> (1)..(681)

<400> 6
atg aag tca tta gct tcg ccg ccg tgt ctc cgc ctg ata ccg acg gca 48
Met Lys Ser Leu Ala Ser Pro Pro Cys Leu Arg Leu Ile Pro Thr Ala
1 5 10 15

cac cgt cag ctc aat tcg cgt caa tct tcc tcc gcc tgt tat ata cac 96
His Arg Gln Leu Asn Ser Arg Gln Ser Ser Ser Ala Cys Tyr Ile His
20 25 30

```
ggt ggc tct tct gtg aac aaa tcc aat aat ctc tca ttc tcc tca tcc   144
Gly Gly Ser Ser Val Asn Lys Ser Asn Asn Leu Ser Phe Ser Ser Ser
         35                  40                  45

aca tcc gga ttt gcg tca cca cta gct gta gag aag gaa tta cgc tct   192
Thr Ser Gly Phe Ala Ser Pro Leu Ala Val Glu Lys Glu Leu Arg Ser
     50                  55                  60

tca ttc gta cag acg gct gct gtt cgc cat gtt acg ggg tct ctt atc   240
Ser Phe Val Gln Thr Ala Ala Val Arg His Val Thr Gly Ser Leu Ile
 65                  70                  75                  80

aga ggc gaa ggt ctt aga ttc gcc atc gtg gta gct cgt ttc aat gag   288
Arg Gly Glu Gly Leu Arg Phe Ala Ile Val Val Ala Arg Phe Asn Glu
                 85                  90                  95

gtt gtg act aag ttg ctt ttg gaa gga gcg att gag act ttc aag aag   336
Val Val Thr Lys Leu Leu Leu Glu Gly Ala Ile Glu Thr Phe Lys Lys
            100                 105                 110

tat tca gtc aga gaa gaa gac att gaa gtt att tgg gtt cct ggc agc   384
Tyr Ser Val Arg Glu Glu Asp Ile Glu Val Ile Trp Val Pro Gly Ser
        115                 120                 125

ttt gaa att ggt gtt gtt gca caa aat ctt ggg aaa tcg gga aaa ttt   432
Phe Glu Ile Gly Val Val Ala Gln Asn Leu Gly Lys Ser Gly Lys Phe
    130                 135                 140

cat gct gtt tta tgt atc ggc gct gtg ata aga gga gat acc aca cat   480
His Ala Val Leu Cys Ile Gly Ala Val Ile Arg Gly Asp Thr Thr His
145                 150                 155                 160

tat gat gct gtt gcc aac tct gct gcg tct gga gta ctt tct gct agc   528
Tyr Asp Ala Val Ala Asn Ser Ala Ala Ser Gly Val Leu Ser Ala Ser
                165                 170                 175

ata aat tca ggc gtt cca tgc ata ttt ggt gta ctg act tgc gag gac   576
Ile Asn Ser Gly Val Pro Cys Ile Phe Gly Val Leu Thr Cys Glu Asp
            180                 185                 190

atg gat cag gct ctg aat cga tct ggt ggc aaa gcc ggc aat aag gga   624
Met Asp Gln Ala Leu Asn Arg Ser Gly Gly Lys Ala Gly Asn Lys Gly
        195                 200                 205

gct gaa act gct ttg acg gcg ctc gaa atg gcg tcg ttg ttt gag cac   672
Ala Glu Thr Ala Leu Thr Ala Leu Glu Met Ala Ser Leu Phe Glu His
    210                 215                 220
```

cac ctg aaa tagctcggct cgttccat 699
His Leu Lys
225

<210> 7
<211> 227
<212> PRT
<213> Arabidopsis thaliana

<400> 7

```
Met Lys Ser Leu Ala Ser Pro Pro Cys Leu Arg Leu Ile Pro Thr Ala
  1               5                  10                  15

His Arg Gln Leu Asn Ser Arg Gln Ser Ser Ser Ala Cys Tyr Ile His
             20                  25                  30

Gly Gly Ser Ser Val Asn Lys Ser Asn Asn Leu Ser Phe Ser Ser Ser
         35                  40                  45

Thr Ser Gly Phe Ala Ser Pro Leu Ala Val Glu Lys Glu Leu Arg Ser
     50                  55                  60

Ser Phe Val Gln Thr Ala Ala Val Arg His Val Thr Gly Ser Leu Ile
 65                  70                  75                  80

Arg Gly Glu Gly Leu Arg Phe Ala Ile Val Val Ala Arg Phe Asn Glu
                 85                  90                  95

Val Val Thr Lys Leu Leu Leu Glu Gly Ala Ile Glu Thr Phe Lys Lys
            100                 105                 110

Tyr Ser Val Arg Glu Glu Asp Ile Glu Val Ile Trp Val Pro Gly Ser
        115                 120                 125

Phe Glu Ile Gly Val Val Ala Gln Asn Leu Gly Lys Ser Gly Lys Phe
    130                 135                 140

His Ala Val Leu Cys Ile Gly Ala Val Ile Arg Gly Asp Thr Thr His
145                 150                 155                 160

Tyr Asp Ala Val Ala Asn Ser Ala Ala Ser Gly Val Leu Ser Ala Ser
                165                 170                 175

Ile Asn Ser Gly Val Pro Cys Ile Phe Gly Val Leu Thr Cys Glu Asp
            180                 185                 190
```

```
Met Asp Gln Ala Leu Asn Arg Ser Gly Gly Lys Ala Gly Asn Lys Gly
        195                 200                 205

Ala Glu Thr Ala Leu Thr Ala Leu Glu Met Ala Ser Leu Phe Glu His
    210                 215                 220

His Leu Lys
225
```

<210> 8
<211> 114
<212> PRT
<213> Arabidopsis thaliana

<400> 8

```
Met Lys Ser Leu Ala Ser Pro Pro Cys Leu Arg Leu Ile Pro Thr Ala
  1               5                  10                  15

His Arg Gln Leu Asn Ser Arg Gln Ser Ser Ser Ala Cys Tyr Ile His
             20                  25                  30

Gly Gly Ser Ser Val Asn Lys Ser Asn Asn Leu Ser Phe Ser Ser Ser
         35                  40                  45

Thr Ser Gly Phe Ala Ser Pro Leu Ala Val Glu Lys Glu Leu Arg Ser
     50                  55                  60

Ser Phe Val Gln Thr Ala Ala Val Arg His Val Thr Gly Ser Leu Ile
 65                  70                  75                  80

Arg Gly Glu Gly Leu Arg Phe Ala Ile Val Val Ala Arg Phe Asn Glu
                 85                  90                  95

Val Val Thr Lys Leu Leu Leu Glu Gly Ala Ile Glu Thr Phe Lys Lys
            100                 105                 110

Tyr Ser
```

<210> 9
<211> 114
<212> PRT
<213> Arabidopsis thaliana

<400> 9

```
Val Arg Glu Glu Asp Ile Glu Val Ile Trp Val Pro Gly Ser Phe Glu
 1               5                  10                  15

Ile Gly Val Val Ala Gln Asn Leu Gly Lys Ser Gly Lys Phe His Ala
            20                  25                  30

Val Leu Cys Ile Gly Ala Val Ile Arg Gly Asp Thr Thr His Tyr Asp
        35                  40                  45

Ala Val Ala Asn Ser Ala Ala Ser Gly Val Leu Ser Ala Ser Ile Asn
    50                  55                  60

Ser Gly Val Pro Cys Ile Phe Gly Val Leu Thr Cys Glu Asp Met Asp
65                  70                  75                  80

Gln Gln Ala Leu Asn Arg Ser Gly Gly Lys Ala Gly Asn Lys Gly Ala
                85                  90                  95

Glu Thr Ala Leu Thr Ala Leu Glu Met Ala Ser Leu Phe Glu His His
            100                 105                 110

Leu Lys
```

<210> 10
<211> 156
<212> PRT
<213> Escherichia coli

<400> 10

```
Met Asn Ile Ile Glu Ala Asn Val Ala Thr Pro Asp Ala Arg Val Ala
 1               5                  10                  15

Ile Thr Ile Ala Arg Phe Asn Asn Phe Ile Asn Asp Ser Leu Leu Glu
            20                  25                  30

Gly Ala Ile Asp Ala Leu Lys Arg Ile Gly Gln Val Lys Asp Glu Asn
        35                  40                  45

Ile Thr Val Val Trp Val Pro Gly Ala Tyr Glu Leu Pro Leu Ala Ala
    50                  55                  60

Gly Ala Leu Ala Lys Thr Gly Lys Tyr Asp Ala Val Ile Ala Leu Gly
65                  70                  75                  80

Thr Val Ile Arg Gly Gly Thr Ala His Phe Glu Tyr Val Ala Gly Gly
                85                  90                  95
```

```
Ala Ser Asn Gly Leu Ala His Val Ala Gln Asp Ser Glu Ile Pro Val
            100                 105                 110

Ala Phe Gly Val Leu Thr Thr Glu Ser Ile Glu Gln Ala Ile Glu Arg
        115                 120                 125

Ala Gly Thr Lys Ala Gly Asn Lys Gly Ala Glu Ala Ala Leu Thr Ala
    130                 135                 140

Leu Glu Met Ile Asn Val Leu Lys Ala Ile Lys Ala
145                 150                 155
```

<210> 11
<211> 42
<212> PRT
<213> Bacillus subtilis

<400> 11

```
Met Asn Ile Ile Gln Gly Asn Leu Val Gly Thr Gly Leu Lys Ile Gly
  1               5                  10                  15

Ile Val Val Gly Arg Phe Asn Asp Phe Ile Thr Ser Lys Leu Leu Ser
             20                  25                  30

Gly Ala Glu Asp Ala Leu Leu Arg His Gly
         35                  40
```

<210> 12
<211> 112
<212> PRT
<213> Bacillus subtilis

<400> 12

```
Val Asp Thr Asn Asp Ile Asp Val Ala Trp Val Pro Gly Ala Phe Glu
  1               5                  10                  15

Ile Pro Phe Ala Ala Lys Lys Met Ala Glu Thr Lys Lys Tyr Asp Ala
             20                  25                  30

Ile Ile Thr Leu Gly Thr Val Ile Arg Gly Ala Thr Thr His Tyr Asp
         35                  40                  45

Tyr Val Cys Asn Glu Ala Ala Lys Gly Ile Ala Gln Ala Ala Asn Thr
     50                  55                  60
```

```
Thr Gly Val Pro Val Ile Phe Gly Ile Val Thr Thr Glu Asn Ile Glu
 65                  70                  75                  80

Gln Ala Ile Glu Arg Ala Gly Thr Lys Ala Gly Asn Lys Gly Val Asp
                 85                  90                  95

Cys Ala Val Ser Ala Ile Glu Met Ala Asn Leu Asn Arg Ser Phe Glu
            100                 105                 110
```